DEPRESSION OF THE ICE-NUCLEATION TEMPERATURE OF RAPIDLY COOLED MOUSE EMBRYOS BY GLYCEROL AND DIMETHYL SULFOXIDE

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ABSTRACT The temperature at which ice formation occurs in supercooled cytoplasm is an important element in predicting the likelihood of intracellular freezing of cells cooled by various procedures to subzero temperatures. We have confirmed and extended prior indications that permeating cryoprotective additives decrease the ice nucleation temperature of cells, and have determined some possible mechanisms for the decrease. Our experiments were carried out on eight-cell mouse embryos equilibrated with various concentrations (0–2.0 M) of dimethyl sulfoxide or glycerol and then cooled rapidly. Two methods were used to assess the nucleation temperature. The first, indirect, method was to determine the in vitro survival of the rapidly cooled embryos as a function of temperature. The temperatures over which an abrupt drop in survival occurs are generally diagnostic of the temperature range for intracellular freezing. The second, direct, method was to observe the microscopic appearance during rapid cooling and note the temperature at which nucleation occurred. Both methods showed that the nucleation temperature decreased from −10 to −15°C in saline alone to between −38° and −44°C in 1.0–2.0 M glycerol and dimethyl sulfoxide. The latter two temperatures are close to the homogeneous nucleation temperatures of the solutions in the embryo cytoplasm, and suggest that embryos equilibrated in these solutions do not contain heterogeneous nucleating agents and are not accessible to any extracellular nucleating agents, such as extracellular ice. The much higher freezing temperatures of cells in saline or in low concentrations of additive indicate that they are being nucleated by heterogeneous agents or, more likely, by extracellular ice.

INTRODUCTION

Injury to cells during cooling to and warming from subzero temperatures is related to the fate of their intracellular water during cooling. As a cell suspension is cooled, ice first forms in the extracellular solution but is unable to cross the cell membrane. As a consequence, the cytoplasm supercools. If the rate of cooling is sufficiently low, the intracellular water will flow out of the cell and freeze extracellularly. But if the rate is too high, the cytoplasm will not have sufficient time to lose water; it will become increasingly supercooled and will eventually freeze. Formation of these intracellular ice crystals and their subsequent growth by recrystallization during warming almost always leads to injury (Mazur, 1965, 1977).

A quantitative description of the movement of intracellular water during cooling has enabled the calculation of the extent to which the cytoplasm of a cell is supercooled at any temperature (Mazur, 1963 *a*, 1966). Such calculations, in turn, permit prediction of the likelihood of ice forming in the cell during cooling at any particular rate. A critical parameter in making such predictions is the temperature at which the supercooled cytoplasm is assumed to freeze (Mazur, 1977). Scattered observations suggest that its ice-nucleation temperature lies between −10 and −20°C (Mazur, 1965). This conclusion has been confirmed by direct microscopic observations in human red cells and HeLa tissue culture cells in the absence of cryoprotective additives (Luyet and Pribor, 1965; McGrath, 1974 and unpublished manuscript), and indi-

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rectly in lymphocytes by survival studies (Knight et al., 1977; McGann, 1978).

It has been generally assumed that this range of nucleation temperatures of $-10°$ to $-20°C$ applies regardless of the presence or absence of cryoprotective additives, and that as long as some freezable water is present, the range is unaffected by the extent of prior dehydration (see Mazur, 1966). However, recent data by Leibo, 1977 and Leibo et al., 1978 indicate that the ice-nucleation temperature of unfertilized mouse ova cooled rapidly in the presence of dimethyl sulfoxide (DMSO) or glycerol can be depressed to temperatures as low as $-40°C$.

In the current study, we have extended Leibo's initial work to eight-cell mouse embryos and to a wider range of DMSO and glycerol concentrations. The additional information permits one to draw conclusions as to the mechanisms responsible for intracellular nucleation and its supression by additives.

We used two methods to detect the occurrence of intracellular freezing, an indirect one and a direct one. The first, indirect, method rested on the assumption, already demonstrated in several cell types (Mazur, 1977), that the death of cells cooled at rates well above optimum is closely coupled with, and probably due to, intracellular freezing. The optimum rate for mouse embryos is ~0.5°C/min. Our experiments accordingly used a cooling rate of 18–20°C/min. In the second, direct, method the formation of intracellular ice was observed by light microscopy during cooling and warming on a specialized low-temperature microscope stage (Diller et al., 1972, McGrath et al., 1975).

The observed nucleation temperature was then compared with that expected if nucleation resulted from homogeneous nucleation. The latter was based on an empirical relationship found by Rasmussen and MacKenzie (1972). The comparison leads to two conclusions: (*a*) Intracellular-ice nucleation in the absence of DMSO or glycerol is by a heterogeneous mechanism, but (*b*) in the presence of increasing concentrations of either cryoprotectant, the nucleation temperature is increasingly depressed, until finally in 1.5 and 2 M concentrations it drops close to the temperature expected for nucleation by a homogeneous mechanism.

THEORY AND CALCULATIONS

Nucleation of Supercooled Solutions

There are three mechanisms by which the freezing of supercooled solutions is initiated. The first is homogeneous nucleation. In this mechanism, the water molecules spontaneously aggregate to form ice nuclei that are large enough to grow spontaneously. The second mechanism is heterogeneous nucleation. Here the aggregation process is catalyzed by insoluble foreign material (dust particles, container wall, etc.). Nucleation theory describes the rate of formation of these small ice nuclei. The classical thermodynamic approach was first proposed by Volmer and Weber (1926) for condensation from vapors, and it was later applied to crystallization from liquids by Turnbull and Fisher (1949). Detailed expositions of the thermodynamics of nucleation have been given elsewhere (see Fletcher, 1962; Walton, 1969). The third mechanism, a special case of heterogeneous nucleation, is the nucleation of supercooled water by contact with an exogenous ice crystal. This occurs, for example, when one "seeds" a supercooled aqueous solution with a small crystal of ice.

Rasmussen and MacKenzie (1972) have studied the first mechanism, homogeneous nucleation, in various aqueous solutions of cryobiological interest by using differential thermal analysis. The contributions of random heterogeneous nucleators were minimized by isolating them in droplets in emulsions of the solutions in an insoluble carrier fluid (e.g., heptane or silicone oil). Upon examining a number of solutes they discovered an empirical relationship between the nucleation temperature (T_n) and the equilibrium melting point (T_f) of the droplets. Low molecular weight solutes were found to depress T_n by a factor of 2 ± 0.2 times the freezing point depression (ΔT_f). This relationship permits one, then, to calculate the T_n of cytoplasm when the cytoplasm contains various concentrations of cryoprotective additives. Such calculations for the additives used in our study on eight-cell mouse embryos will now be considered.

Calculation of the Homogeneous Nucleation Temperature of Mouse Embryos

Assumptions. Calculation of the homogeneous nucleation temperature of eight-cell mouse embryos assumed first that the cytoplasm has the same nucleation kinetics as an isotonic, 0.9% (wt/vol) NaCl solution (Maffly and Leaf, 1958). Second, the depression of T_n of the cytoplasm was assumed to be the sum of depressions due to the cytoplasmic solutes and the intracellular cryoprotective additive (if present). In other words, each cell was assumed to have the same nucleation behavior as an emulsion of equivalent-sized droplets of saline plus cryoprotectant in an inert oil.

Procedure. The homogeneous nucleation temperature of a cell should depend on its size and on the concentration of solutes in its cytoplasm. Wood and Walton (1970) have examined the effect of droplet size. They noted that a tenfold increase in the droplet diameter increases the value of T_n by 2°C. If this relationship is combined with the published value of approximately $-39°C$ for the homogeneous nucleation temperature for a 1-μm-diameter water droplet (Thomas and Staveley, 1952; Mossop, 1955), one predicts a T_n of about $-36°C$ for a droplet of water 47 μm in diameter, the size of a blastomere of an eight-cell mouse embryo.

The depression of T_n due to the solute in the cytoplasm can be calculated from the relation derived by Rasmussen and Mackenzie (1972); namely,

$$T_n = -36 - (2 \times \text{total } \Delta T_f), \quad (1)$$

where -36 is the T_n of a water droplet with a diameter of 47 μm, and (total ΔT_f) is the freezing point depression of the cytoplasmic solutes plus additive. The results of such a calculation for embryos equilibrated in glycerol or DMSO are shown in Table I.

MATERIALS AND METHODS

Embryo Isolation

Eight-cell–stage mouse embryos were obtained from F_1 [C3D2F_1:jax] virgin females (6–15 weeks old) mated with F_1 males [B6D2F_1:jax]. The females were induced to superovulate by the intraperitoneal injection of 5–7.5 IU of gonadotropins from the serum of pregnant mares (Sigma

TABLE I
CALCULATED HOMOGENEOUS NUCLEATION TEMPERATURES OF EIGHT-CELL MOUSE EMBRYOS

Cryoprotective additive concentration	Freezing point depression (ΔTf)			Calculated homogeneous nucleation temperature§ T_n
	Additive*	Cytoplasmic solutes‡	Total	
(M)		(°C)		(°C)
0	0	0.5	0.5	−37
0.5	0.9	0.5	1.4	−39
1.0	1.9	0.5	2.4	−41
1.5	2.8	0.5	3.3	−43
2.0	3.7	0.5	4.2	−45

*1.86 × M.
‡Assuming cytoplasm is equivalent to a 0.85% (wt/vol) solution of NaCl.
§$-36 - (2 \times \text{total } \Delta T_f)$.

Chemical Co., St. Louis MO) and 5–7.5 IU of human chorionic gonadotropins (Sigma Chemical Co.) given 46–48 h apart. The females were mated individually at the time of the chorionic gonadotropin injection, and eight-cell embryos were isolated from their reproductive tracts 66–70 h later. Embryos were flushed from the reproductive tract, pooled, and washed with a modified Dulbecco's phosphate-buffered saline solution (PB1; Whittingham, 1971, 1974). After washing, the embryos were held at ~4°C until used some 10–120 min later.

Freezing Procedure

Solutions. Samples consisted of 10–20 embryos in each of several 10 × 100 mm Pyrex test tubes containing 0.1 ml PB1 at room temperature. An equal volume of PB1 containing 0, 1.0, 2.0, 3.0, or 4.0 M glycerol or DMSO was then added and mixed. The resulting embryo suspensions were held at room temperature for 20–25 min to ensure complete permeation of the additive into the embryos (Mazur et al., 1976; Rall, 1979). Each experimental set consisted of duplicate embryo tubes and a third tube containing the suspending medium and a thermocouple.

Seeding, Cooling, and Thawing. Lucite holders with the three tubes attached were transferred into an ethanol bath precooled ~1–2°C below the freezing point of the suspending solution. After a 2–min equilibration at that temperature, the suspensions were seeded with an ice crystal in the tip of a Pasteur pipette. Seeded samples were held in the bath an additional 4 min while the crystallizing solution equilibrated, and were then transferred to a rapidly stirred ethanol bath previously adjusted to be ~1°C colder than the seeding bath. The bath consisted of a 90 × 290 mm Pyrex tube containing 900 ml of 95% ethanol. The ethanol bath was cooled at ~18° (15°–20°) C/min by immersion into a 4-liter wide-mouth Dewar flask containing 1 liter of liquid nitrogen. The temperature of one sample in each set and that of the bath were continously monitored by a 36 gauge copper-constantan thermocouple attached to a Speedomax Type W recorder (Leeds and Northrop, North Wales, PA).

When the temperature of the embryo suspension had fallen to an appropriate level, the sample was immediately warmed by immersion in an ice-water mixture (warming rate: ~100°C/min). Thawed samples were kept in the ice bath until the samples subjected to the lowest temperatures had been similarly warmed.

Controls consisted of embryos treated as above, except they were not frozen and thawed.

Dilution of Suspending Medium. The presence of intracellular additive makes the embryos susceptible to injury by osmotic shock during dilution. To reduce osmotic shock, two alternative methods were used to dilute the embryos out of the cryoprotectant-PB1 solutions in which they were frozen. Suspensions in DMSO were diluted by a number of small additions of PB1 at ~0°C (Whittingham et al., 1972; Leibo and Mazur, 1978). Suspensions in 0.5 or 1.0 M glycerol were diluted by a second method: the embryos were placed into 0.45 or 0.9 M sucrose in PB1 (Mazur and Miller, 1976; Mazur and Cacheiro, 1977; Leibo and Mazur, 1978; Jackowski et al., 1980). This method relies on the fact that the embryos are impermeable to sucrose. The embryo-sucrose suspension was mixed thoroughly and held at room temperature for 45–60 min. The concentration of sucrose was then reduced by five successive twofold dilutions with PB1 at 2-to 4-min intervals.

Finally, the embryos were recovered from the diluted samples and washed with PB1. Generally, ~90% of the embryos initially placed in a sample tube were recovered after these two steps. The others were lost. The recovered embryos were then assayed for survival.

Survival Assays. The survival of embryos after freezing in DMSO or in 0.5 or 1.0 M glycerol, thawing, and dilution was determined by a standard in vitro culture technique (Whittingham, 1971): The embryos were cultured in a small drop of Brinster's medium for ovum culture (BMOC-3) (Brinster, 1963) in the well of a microtest tissue-culture plate (Falcon No. 3034; Becton, Dickinson and Co., Cockeysville, MD) under washed silicone oil (200 fluid, Dow Corning Corp., Midland, MI). Survival was defined as the percentage of recovered embryos that developed to the expanded blastocyst stage.

We had intended to use a similar assay of survival for embryos frozen in 1.5 and 2.0 M glycerol, but we found that the resulting survivals of both unfrozen controls and frozen-thawed embryos were variable, possibly as a result of osmotic damage during dilution by sucrose. Consequently, in these two concentrations of glycerol we assessed the survival in undiluted samples with a fluorescence technique. This viability assay was first described by Rotman and Papermaster (1965) and has been previously applied to determining the viability of frozen–thawed HeLa cells by McGrath et al. (1975) and of mouse ova by Jackowski et al. (1980). The basic principle of the assay is the following. Nonfluorescent fluorescein diacetate (FDA) permeates the embryos and is then converted enzymatically into fluorescein, an impermeable fluorescent dye. A cell that exhibits fluorescence must possess a membrane that is sufficiently intact to prevent the loss of both intracellular fluorescein and the esterases that cleave the acetate groups from FDA. We carried out the assay as follows: A stock solution of 1.2×10^{-2} M FDA in acetone was stored in the dark at −20°C. Just before use, the stock was diluted 1:100 with isotonic saline and then diluted an additional 1:10 with the appropriate glycerol-PB1 solution to produce 1.2×10^{-5} M (~4 μg/ml) FDA in either 1.5 or 2.0 M glycerol. Frozen-thawed embryos were placed into the appropriate solution for~10 min at room temperature and then observed through a Zeiss Photomicroscope III equipped for fluorescein-isothiocyanate (FITC) fluorescence (Carl Zeiss, Inc., New York, NY). Survival was defined as the percentage of the recovered embryos that exhibited fluorescence in five or more blastomeres (Tarkowski and Wroblewska, 1967).

Low-Temperature Microscopy

The appearance of embryos during cooling and warming was observed by phase-contrast light microscopy on a low-temperature microscope stage described previously (McGrath, 1974; McGrath et al., 1975). The present version of the stage is the same as that used by Leibo et al. (1978) with one modification; namely, the foil thermocouple was sealed between the quartz heater and a 22-mm square cover glass with a thin layer of RTV silicone rubber adhesive (General Electric Corp., Waterford, NY). Embryo suspensions were placed on the cover glass directly over the thermocouple and immediately covered with a 13-mm square cover glass supported at opposite edges by a piece of thin acrylic tape. The

microscope stage was mounted on a Zeiss Universal Research microscope (Carl Zeiss, Inc.) fitted with a long working-distance condenser, and was positioned so that two to nine embryos were visible in the camera field within 350 μm of the thermocouple junction. Three photographs were taken at room temperature. The embryos were then cooled to about +10°C, photographed, and held at this temperature for several minutes while the microscope stage was prepared for controlled cooling. Cooling at 20°C/min was then started, and the embryos were photographed at regular intervals and also whenever one or more blastomeres were observed to undergo intracellular freezing. Intracellular freezing was judged to occur when the blastomeres abruptly blackened or "flashed." The flashing is a result of light scattering by the many small intracellular ice crystals (Smith, 1961) or possibly by small gas bubbles released into solution (Steponkus and Dowgert, 1981). Cooling was continued until all the embryos had frozen intracellularly. The stage was then warmed at ~100°C/min, and the embryos were photographed after thawing. The nucleation temperature of a given embryo was defined as the temperature at which four or more blastomeres flashed. We chose this criterion because Tarkowski and Wroblewska (1967) have shown that more than four intact blastomeres are required for normal embryonic development.

The embryos were photographed at 125 magnification and at constant exposure time in a given run on Kodak Tri-X film with a motor-driven Nikon-F2 35-mm camera. Sample temperatures (±2°C) were continuously recorded on a potentiometric recorder, and an event marker recorded the time of each photograph.

RESULTS

Survival of Embryos Frozen in the Absence and Presence of DMSO Solutions

Figs. 1*a* to *e*, respectively, show the percentage of eight-cell mouse embryos that survived rapid cooling to various subzero temperatures when suspended in PB1 (alone) and in PB1 containing 0.5, 1.0, 1.5, and 2.0 M DMSO. Each figure represents the pooled results of three to five separate experiments. When embryos were cooled in PB1, survival remained high (>80%) until about −10°C, and then decreased to below 10% with decreasing temperature. The drop in survival occurred chiefly between −10° and −18°C, and none of the embryos survived cooling below −30°C. When DMSO was present in the medium, death occurred over a progressively narrower range of temperatures as the DMSO concentration was increased from 0.5 to 2 M. For example, in 0.5 and 2.0 M DMSO, death occurred over a temperature range of 16°C and 2°C, respectively. A second effect of increasing the DMSO concentration was to shift the lethal range to lower temperatures. For instance, the median lethal temperatures (LT_{50}) in 0.5 and 2.0 M DMSO were −31 and −42°C, respectively. The temperatures which killed 90% of the embryos (LT_{90}) frozen in PB1 and PB1 containing 0.5, 1.0, 1.5, and 2.0 M DMSO were −17, −38, −39, −42, and −44°C, respectively.

Survival of Embryos Frozen in Glycerol Solutions

Figs. 2*a* and *b* show the percentages of embryos that survived rapid cooling to various subzero temperatures when suspended in 0.5 and 1.0 M glycerol. Survival was determined by in vitro development after removal of the glycerol by the sucrose dilution technique. Figs. 3*a* and *b* show the comparable percentage survivals for embryos cooled in 1.5 and 2.0 M glycerol. As noted above, these survivals were estimated by the FDA assay in the presence of glycerol. Overall, the response of embryos in glycerol was parallel to that found in the DMSO solutions.

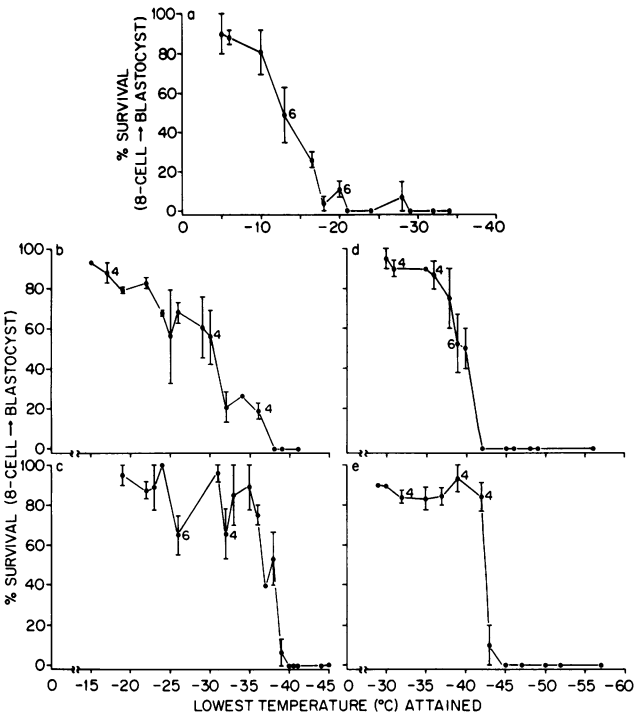

FIGURE 1 Temperature dependence of the survival of eight-cell mouse embryos suspended in PB1 or in DMSO solutions in PB1. The embryos were equilibrated in the appropriate concentration of DMSO, seeded, cooled to the indicated temperature at 20°C/min, and then rapidly thawed. Survival was measured by in vitro development. Each curve here and in Figs. 2 and 3 is based on four separate but overlapping experiments. Each point represents the mean of at least duplicate samples (10–15 embryos/sample). Points based on four or six samples are identified by an accompanying number. Error bars are standard errors. Embryos frozen in (*a*) PB1 or in PB1 plus (*b*) 0.5, (*c*) 1.0, (*d*) 1.5, or (*e*) 2.0 M DMSO.

Comparison of Embryo Survival and the Calculated Homogeneous Nucleation Temperature

We have outlined the reasons for believing that the temperature at which rapidly cooled embryos are killed is a reflection of the temperature at which they freeze intracellularly. This formation of intracellular ice can be induced by either a homogeneous or a heterogeneous nucleation mechanism. The calculated homogeneous nucleation temperatures (T_n) for embryos equilibrated in various concentrations of additives were tabulated in Table I and are plotted in Fig. 4. Fig. 4 also shows the LT_{50}'s and LT_{90}'s for embryos equilibrated in these same concentrations of

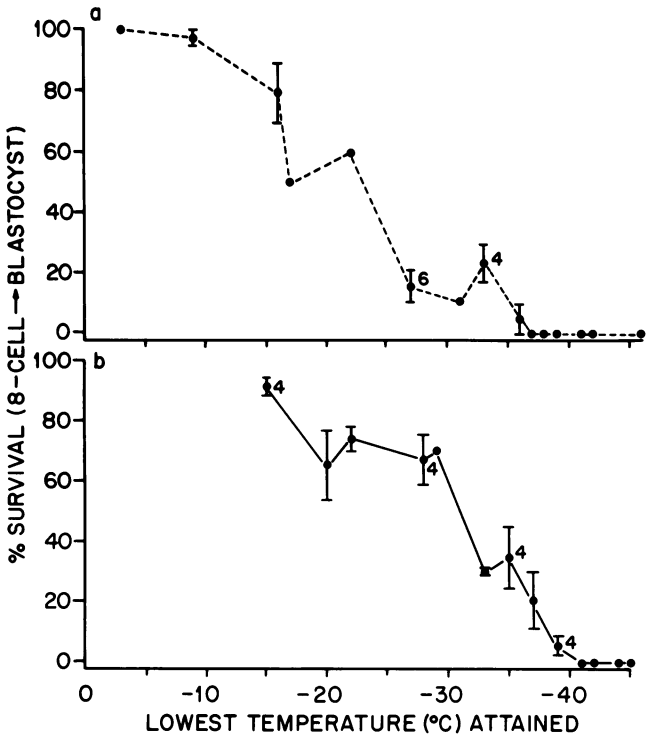

FIGURE 2 Temperature dependence of the survival of eight-cell mouse embryos suspended in 0.5 or 1.0 M glycerol in PB1. The embryos were equilibrated in the appropriate concentration of glycerol, seeded, cooled to the indicated temperature at 20°C/min, and then rapidly thawed. Survival was measured by in vitro development. Embryos frozen in (*a*) 0.5 or (*b*) 1.0 M glycerol.

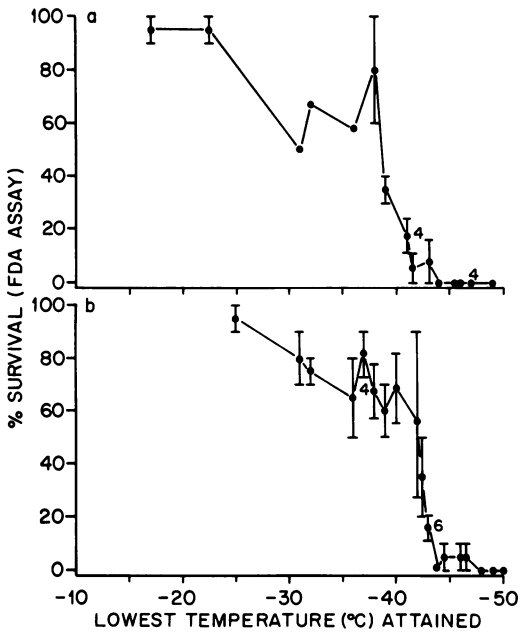

FIGURE 3 Temperature dependence of the survival of eight-cell mouse embryos suspended in 1.5 or 2.0 M glycerol in PB1. The embryos were equilibrated in the appropriate concentration of glycerol, seeded, cooled to the indicated temperature, and then rapidly thawed. Survival was measured by an FDA fluorescence assay (see text for details). Embryos frozen in (*a*) 1.5 or (*b*) 2.0 M glycerol.

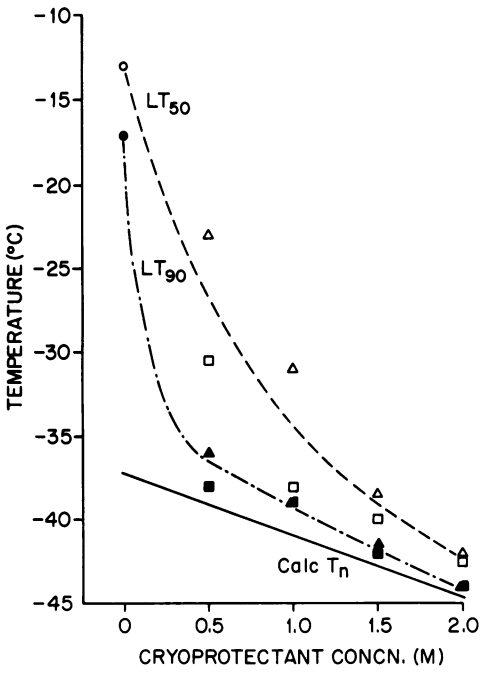

FIGURE 4 Comparison of the calculated homogeneous nucleation temperature (T_n) and the observed temperature that kills 50 and 90% of the embryos (LT_{50} and LT_{90}). The calculated T_n's (Table I) are shown by the solid line. Circles, squares, and triangles refer to embryos in PB1, DMSO, and glycerol, respectively. Open symbols, LT_{50}'s; closed symbols, LT_{90}'s.

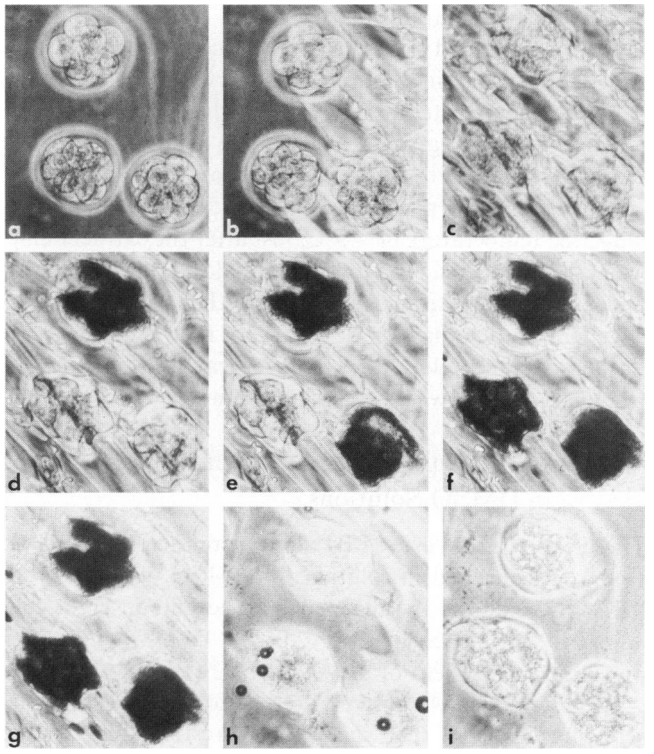

FIGURE 5 Microscopic appearance of eight-cell mouse embryos in PB1 during freezing and thawing. The embryos were cooled at 20°C/min from +10 (*a*) to −3.5 (*b*), −5 (*c*), −9 (*d*), −11.5 (*e*), −15 (*f*), and −24°C (*g*). They were then warmed at ~100°C/min to ~ −3 (*h*) and +25°C (*i*). The unfrozen embryos are ~75 μm in diameter.

additive and cooled at 18°C/min. As the concentration of additive was increased, the LT_{50} and LT_{90} curves approach the T_n curve. This suggests that the heterogeneous nucleators of the embryos either were destroyed or became less effective as the concentration of additive was increased. In other words, because embryo death in the absence of additive occurred at temperatures far above T_n, nucleation of the supercooled cytoplasm was presumably a result of heterogeneous nucleation. On the other hand, when high concentrations of additive (1.5 and 2.0 M) were present, killing occurred over a very narrow temperature range close to the T_n. In this case, then, nucleation of the supercooled cytoplasm appears to have been by a homogeneous mechanism. When intermediate concentrations of additive were present, both mechanisms appear to have operated.

Microscopic Observations on Intracellular Freezing of Embryos Frozen in the Absence of Additives

Our inference that killing was a reflection of intracellular freezing was tested by direct observation of the responses of embryos during cooling. Three eight-cell mouse embryos during cooling at 20°C/min in PB1 are shown in Fig. 5. Ice first appeared at about −3°C (Fig. 5*b*), and then completely surrounded and partly obscured the embryos (Fig. 5*c*). As the temperature was lowered to −9°C, one embryo suddenly blackened as its intracellular contents froze (Fig. 5*d*). The remaining embryos underwent intracellular freezing when the temperature was reduced to −11 (Fig. 5*e*) and −15°C (Fig. 5*f*). After cooling to a final temperature of −24°C (Fig. 5*g*), the embryos were thawed at ~100°C/min. The photograph in Fig. 5*h* was taken at an indicated temperature of −3°C, just after the extracellular ice had disappeared. The embryos had lost their blackened appearance a few seconds before, and they were obviously degenerate after thawing (Fig. 5*i*). In fact, embryos that flashed during cooling always degenerated after thawing whether additives were present or absent.

Intracellular Freezing of Embryos Frozen in DMSO Solutions

These experiments using PB1 alone were then repeated on embryos suspended in solutions of DMSO or glycerol in PB1. Fig. 6 shows the morphological events in eight-cell embryos during cooling in 2.0 M DMSO. Ice first appeared in the field of view at −8°C (Fig. 6*b*), and by −10°C it completely obscured the embryos (Fig. 6*c*). The sequence of photos then shows an interesting step-by-step nucleation of one embryo: Pairs of blastomeres of this embryo flashed at −28.5, −31, −33, and −36.5°C (Fig. 6*d*, 6*e*, 6*f*, and 6*h*, respectively). The blastomeres of the other embryos flashed simultaneously (the more common finding); one embryo each flashed at −35, −44.5, and −45.5°C (Fig. 6*g*, 6*k*, and 6*l*), and two embryos flashed at −39°C.

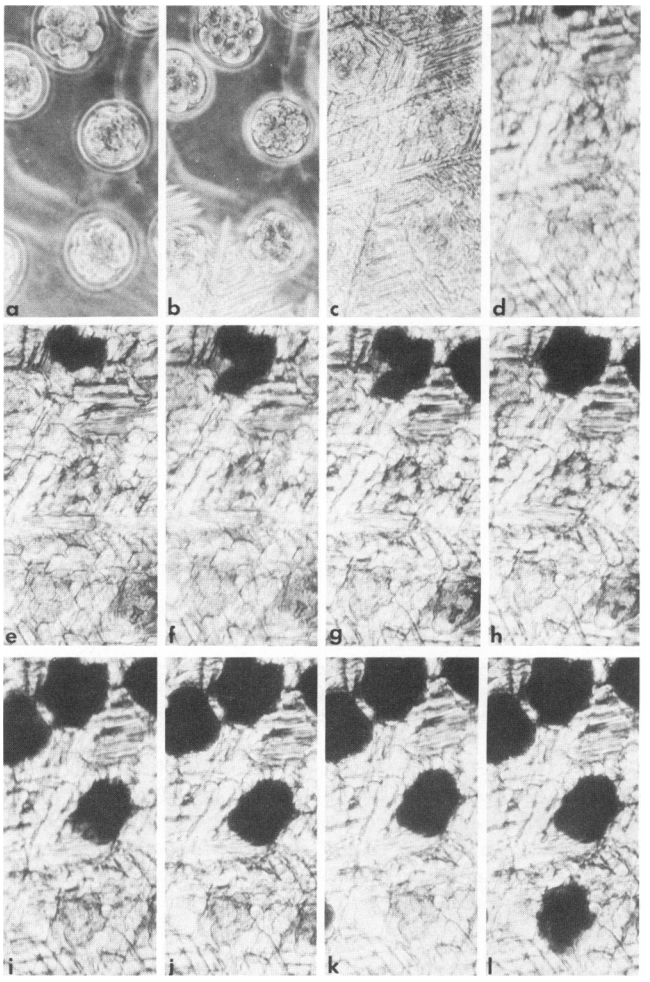

FIGURE 6 Microscopic appearance of eight-cell mouse embryos suspended in 2.0 M DMSO during freezing. The embryos were cooled at 20°C/min from +10 to −7 (*a*), −8 (*b*), −10 (*c*), −28.5 (*d*), −31 (*e*), −33 (*f*), −35 (*g*), −36.5 (*h*), −39 (*i*), −43 (*j*), −44.5 (*k*), and −45.5°C (*l*).

From a number of such serial photographs, we determined the percentages of embryos that underwent intracellular freezing at various temperatures when suspended in 0, 0.5, 1.0, 1.5 or 2.0 M DMSO. The results are given graphically in Fig. 7. As the concentration of DMSO was increased from 0 to 1.5 or 2.0 M, the nucleation curves shift to progressively lower temperatures. (Note that we have data for only seven embryos in 2.0 M DMSO; hence the exact shape and position of that curve is less certain.)

Intracellular Freezing of Embryos in Glycerol Solutions

We then carried out similar experiments on embryos suspended in glycerol-PB1 solutions. The results of these microscopic observations have been collated in Fig. 8 for embryos cooled at 20°C/min in four concentrations of

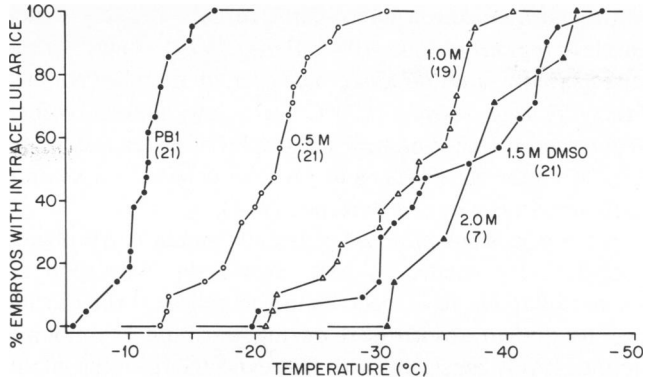

FIGURE 7 Nucleation temperatures of eight-cell mouse embryos suspended in various DMSO-PB1 solutions. The percentages of embryos with intracellular ice are plotted as a function of the temperature during cooling at 20°C/min. The values in parentheses are the total number of embryos observed during cooling in that concentration of DMSO.

glycerol and in PB1 alone. The nucleation behavior in glycerol solutions is qualitatively similar to that of embryos frozen in DMSO solutions (compare Figs. 7 and 8). For example, high concentrations of glycerol (1.0, 1.5, and 2.0 M) depressed the nucleation temperature more than a low concentration (0.5M). The primary difference between the two additives is that embryos cooled in glycerol usually underwent intracellular freezing at a slightly higher temperature than did embryos cooled in the same concentration of DMSO. However, because fewer embryos were observed in each glycerol solution (8–13), the observed positions of the glycerol nucleation curves are subject to greater statistical uncertainty.

Correlation between Killing and Intracellular Freezing

Table II shows that the temperatures at which we observed flashing in eight-cell embryos correlated reasonably well

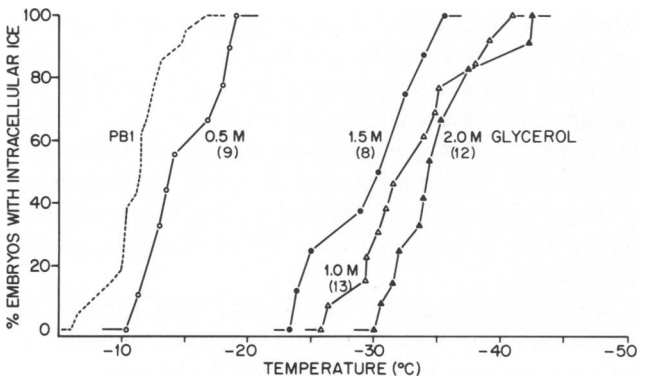

FIGURE 8 Nucleation temperatures of eight-cell mouse embryos suspended in various glycerol-PB1 solutions. The percentages of embryos with intracellular ice are plotted as a function of the temperature during cooling at 20°C/min. The values in parentheses are the total number of embryos observed during cooling in that concentration of glycerol.

TABLE II
COMPARISON BETWEEN THE OBSERVED INTRACELLULAR NUCLEATION TEMPERATURE OF EIGHT-CELL MOUSE EMBRYOS AND THEIR CALCULATED HOMOGENEOUS NUCLEATION TEMPERATURE

Additive	Concentration	Killing temperature		Observed temperature for		Calculated homogeneous nucleation temperature, T_n
		LT_{50}	LT_{90}	50% Frozen Cells	90% Frozen Cells	
	(*M*)	(°C)		(°C)		(°C)
None	0	−12	−17	−12	−14	−37
DMSO	0.5	−31	−38	−22	−26	−39
	1.0	−38	−39	−33	−37	−41
	1.5	−40	−42	−34	−43	−43
	2.0	−42	−44	−37	−45	−45
Glycerol	0.5	−23	−36	−14	−18	−39
	1.0	−31	−38	−32	−39	−41
	1.5	−38	−41	−30	−35	−43
	2.0	−42	−44	−35	−42	−45

with the temperatures at which we observed an abrupt drop in survival. The correlation is best in the 1, 1.5, and 2.0 M concentrations of additive. Generally, however, the killing temperatures tended to be several degrees lower than the temperatures that produced "flashing."

Another difference is the temperature range over which the two responses occur. The transition from the absence of intracellular freezing to the intracellular freezing of most or all the embryos occurred over a relatively fixed and narrow temperature range that was independent of the concentration of additive, i.e., the curves in Figs. 7 and 8 are quite parallel. On the other hand, as noted, the temperature range over which the drop in survival occurred became progressively narrower as the additive concentration was increased (Figs. 1 and 2).

DISCUSSION

It has long been known that glycerol can prevent the deleterious effects of slow freezing (Polge et al., 1949). Its protective action is believed to be related to its colligative properties of reducing the increase in the concentration of salts in the unfrozen portion of the suspending solution (Lovelock, 1953; Rall et al., 1978) and of increasing the fraction of the extracellular solution that remains unfrozen at a given temperature (Mazur et al., 1981). DMSO is thought to act in a similar manner (Lovelock and Bishop, 1959). Until recently it was thought that cryoprotective additives only protect against injury when cooling is slow enough to preclude intracellular ice. But recent work suggests that DMSO can protect some cells during rapid cooling to temperatures as low as −40°C even when the cooling rate is high enough to expect the occurrence of intracellular freezing (Farrant, 1977; Leibo et al., 1978).

Leibo (1977) and Leibo et al. (1978) were the first to observe that DMSO and glycerol suppress the nucleation temperature of unfertilized mouse ova, and to note that this suppression might account for the ability of embryos to withstand rapid cooling to as low as −40°C. We have now extended this finding to the eight-cell stage of mouse embryos. Furthermore, our results indicate the basis of the suppression of the nucleation temperature: In the absence of the additives, nucleation occurs between −10 and −15°C. This is so far above the homogeneous nucleation temperature that it must necessarily be catalyzed either by heterogeneous nucleating agents or by extracellular ice. But in the presence of 1.5 or 2.0 M DMSO or glycerol, these heterogeneous agents either no longer exist or are no longer effective. As a consequence, intracellular nucleation has to occur by the spontaneous mechanism of homogeneous nucleation, a mechanism that should operate only below −36°C in objects the size of the blastomeres of the embryos. The temperature of intracellular freezing will be lowered even further because the additives also suppress the homogeneous nucleation temperature itself by about 4 degrees for each mole of additive per liter of solution. Consequently, in 2 M additive solutions, intracellular freezing should occur well below −40°C.

We now discuss the evidence for these conclusions and then evaluate several possible explanations as to why heterogeneous nucleating agents including extracellular ice either cannot penetrate the cell, or are absent or inoperative in the cell in the presence of high concentrations of glycerol and DMSO.

Evidence that "Flashing" of Rapidly Cooled Embryos is a Result of the Formation of Ice in Supercooled Cytoplasm

Embryos (and other cells and tissues) can be observed to undergo a sudden darkening under the microscope when cooled relatively rapidly to subzero temperatures. There is strong circumstantial evidence that such "flashing" is a manifestation of the formation of many small or highly branched intracellular ice crystals:

(*a*) Optical flashing occurs only when cells are cooled at rates that are too high to permit extensive cellular dehydration during cooling (see Mazur, 1977).

(*b*) The temperature at which flashing is observed is usually below −10°C and is always below the freezing point of cytoplasm (−0.6°C for mammalian cells in the absence of additives and about −4.0°C in a 2 M concentration of additive).

(*c*) When the rate of warming is low enough to permit the accurate determination of temperature (less than ~50°C/min), the blackened appearance of the embryos always disappears before or at the expected melting point of the cytoplasm (Rall, 1981; Rall et al., 1980).

(*d*) When rapidly cooled cells are cleaved and replicated at temperatures below −100°C, the replicas, when examined by electron microscopy, reveal relatively structureless regions (Moor, 1964; Bank, 1974). These structures rapidly sublime away, however, if the cleaved surfaces are held above −100°C for a few minutes before replication ("freeze-etched"). Samples of water (i.e., pure ice) and aqueous solutions of glycerol behave in a similar manner (Staehelin and Bertaud, 1971).

(*e*) Suspensions of yeast cells and human erythrocytes cooled under conditions that ensure the formation of intracellular ice have been found to release heat during cooling and to absorb heat during warming in amounts quantitatively consistent with that expected from the latent heat of crystallization and fusion of their intracellular water (Wood and Rosenberg, 1957; Mazur, 1963 *b*; Rasmussen et al., 1975).

Most investigators have assumed that the sudden darkening is a result of light scattering by highly branched intracellular ice crystals. Steponkus and Dowgert (1981) have shown that intracellular ice formation can also cause the release of small gas bubbles, and they ascribe the darkening of frozen cells to light scattering by these bubbles. However, freeze-cleaved cells generally show none of the holes that would be expected if intracellular bubble formation had occurred.

Evidence that the Killing of Rapidly Cooled Embryos is an Indication of Intracellular Freezing

We have assumed in this paper that when the survival of rapidly cooled embryos drops abruptly below a certain temperature, it is diagnostic of the formation of substantial quantities of intracellular ice. This assumption is based on theoretical considerations and on a variety of findings with other cells, chiefly yeast and mammalian erythrocytes (see Mazur, 1977, for a review). In brief, a physical-chemical model, developed by Mazur (1963 *a*) and confirmed and extended by Mansoori (1975) and Silvares et al. (1975), predicts that cells cooled above certain rates become extensively supercooled. As long as the cooling rates are not high enough to induce vitrification, this supercooling has to be eliminated by intracellular ice formation. The cooling rates predicted by this model to have a high probability of causing intracellular freezing are close to the cooling rates that are observed to cause the flashing of cells, and are also close to the cooling rates that result in an abrupt drop in survival below certain threshold subzero temperatures.

We noted in connection with Table II that although the temperatures at which flashing occurred in eight-cell embryos correlate reasonably well with the temperatures over which survival dropped abruptly, the killing temperatures tended to be several degress lower than the flashing temperatures. There are several possible reasons:

(*a*) The embryos assayed for survival were not the actual embryos that were observed under the microscope.

The two sets were treated under parallel conditions, but the sample geometries were different; i.e., 0.2 ml of suspension in the bottom of small tubes vs. thin layers in the microscope slide preparations, respectively. Perhaps the restrictions on ice growth in the latter conditions alter the relationship between embryo and extracellular ice and thereby enhance the heterogeneous nucleation of the supercooled cytoplasm.

(*b*) We have no way of knowing how much time is required to translate the sequelae of flashing into cell death. If the translation time took 15 s, killing would occur some 5°C below flashing because we were cooling the embryos at 20°C/min. Furthermore, although one can calculate the fraction of intracellular water that has to be converted to ice to achieve equilibrium, we do not know what percentage is converted to ice at the observed instant of flashing, nor do we know what percentage has to be converted to ice to cause cell death. The observation that flashing precedes killing is not unique to this study. Leibo et al. (1978) also found this to be the case with respect to cooling rate, and Rall et al. (1980) have reported it with respect to events during warming.

(*c*) We used fairly rapid cooling (20°C/min) and quite rapid warming (~100°C/min). The purpose of the latter was to minimize the possibility of the occurrence of the intracellular freezing of supercooled cells during warming, and to prevent the possibility of deleterious effects from dehydration during slow warming. But such rapid warming can also "rescue" cells that contain intracellular ice, presumably by virtue of its ability to decrease the extent of crystal growth by recrystallization during warming. For example, Asahina and his colleagues have reported that, on the basis of light or electron microscopic criteria, ascites tumor cells that contain intracellular ice will sometimes survive if they are warmed rapidly (Asahina et al., 1970; Shimada and Asahina, 1975).

Factors Responsible for the Suppression of Intracellular Nucleation in Glycerol and DMSO Solutions

Regardless of the correct explanation for the differences between the two assays of intracellular freezing, the fact remains, as shown in Table II, that nucleation occurs far above the homogeneous nucleation temperature in the absence of additives, whereas in the presence of 1.5 or 2.0 M additive it occurs close to the expected homogeneous nucleation temperature. The only explanation appears to be that cell nucleation in the absence of additive is almost wholly a result of heterogeneous nucleating agents but that these agents are either eliminated or rendered ineffective in the presence of molar concentrations of DMSO and glycerol.

Anticipating a point to be discussed shortly, one can word this explanation in a way that emphasizes possible changes in the cell rather than changes in the nucleating agents: Embryos do not normally contain ice-nucleating agents, and, in the presence of 1.5 or 2.0 M additive, any extracellular heterogeneous nucleators cannot penetrate the cell. Consequently, the cell can only nucleate homogeneously. But in the absence of or in low concentrations of additive, the cell surface might become altered in some way so as to allow extracellular nucleators to pass into the supercooled interior.

To understand the functioning of heterogeneous nucleators, we need to know what they might be.

Heterogeneous Nucleation

Extracellular Ice. Mazur (1965, 1966) proposed that the heterogeneous nucleating agent is in fact extracellular ice. According to this view, the cell membrane can act as a barrier to prevent the growth of extracellular ice into supercooled cytoplasm. Below a critical temperature (usually between −10 and −20°C), however, the membrane loses its barrier properties and extracellular ice nucleates the cytoplasm. Several studies indicate that when extracellular ice is absent during cooling, cells undergo intracellular freezing at lower temperatures than they do when extracellular ice is present. On the basis of such observations, Mazur (1966) suggested several possibilities as to why the cell membrane might lose its barrier properties below a certain temperature:

(*a*) the cell membrane only comes in direct contact with extracellular ice below a critical temperature,

(*b*) the ability of ice to grow through the membrane increases with lowered temperature, or

(*c*) the barrier properties of the membrane change with lowered temperature.

One speculative model in the second category holds that only nanometer-sized ice crystals are able to penetrate the aqueous channels that presumably exist in cell membranes. But small ice crystals have high surface energies due to their small radii of curvature and hence can exist only at low temperatures. Consequently, the barrier properties arise because ice crystals small enough to pass through such pores cannot exist above about −10°C.

Intracellular Nucleators. An alternative explanation for the heterogeneous nucleation of supercooled cytoplasm is that the nucleators are endogenous substances in the cell (Toscano et al., 1975). Rasmussen et al. (1975) carried out the first systematic investigation of cytoplasmic nucleators. By preparing water in oil emulsions of solutions containing yeast or human erythrocytes, they showed that the droplets supercooled to temperatures near their expected T_n (below −40°C). Such a result would indicate that the ctyoplasm of yeast or erythrocytes contains no effective nucleators. However, Franks and Bray (1980)have obtained different preliminary results. Using a similar emulsion technique, they report that the nucleation of yeast and soybean cells always occurs at temperatures

above −35°C. But Burke et al. (1975, 1976) have examined still other plant tissues and found that they do not contain heterogeneous nucleators. Indeed, the absence of nucleating agents seems to account for winter hardiness in many species of insects and woody plants, i.e., their tissues can supercool to temperatures as low as −45°C.

Effect of Cryoprotective Additives. Regardless of the precise mechanism by which supercooled cytoplasm is heterogeneously nucleated, we find that the nucleating agents become less effective in embryos in the presence of glycerol or DMSO, and they become almost completely ineffective when the concentration of additive reaches 1.5–2.0 M (Fig. 4 and Table II). There are several possibilities:

(*a*) Cryoprotective additives may reduce the ability of heterogeneous nucleators to catalyse the formation of stable ice nuclei by altering the thermodynamic stability of ice clusters in a manner comparable to that proposed by Rasmussen and Mackenzie (1972) for the effects of solutes on homogeneous nucleation.

(*b*) The presence of glycerol and DMSO could also reduce the effectiveness of extracellular ice as a heterogeneous nucleating agent in a very different way. The higher the concentration of these solutes, the greater will be the fraction of the solution remaining unfrozen at a given subzero temperature (Rall et al., 1978; Mazur et al., 1981), and the lower will be the likelihood that ice will come in direct contact with the cell membrane. Furthermore, direct contact between extracellular ice and the surface of the cell membrane may be more difficult in early embryos than in other cells, because early embryos are surrounded by a nonliving barrier, the zona pellucida, and are separated from the zona by the perivitelline space. As freezing progresses, cells are restricted to ever narrowing channels of unfrozen liquid bordered by plates of ice. The zona will tend to shield the embryo protoplast from direct contact with the surrounding ice. The zona is too porous to block the eventual growth of ice crystals into the perivitelline space, but it could delay such growth to a lower temperature.

(*c*) A third possibility is that additives could lower the nucleation temperature to near −40°C by preventing changes in membrane architecture that would permit the passage of extracellular ice crystals at higher temperatures. If this explanation is valid, intracellular freezing at higher temperatures would be a consequence of prior membrane changes and not, as is generally argued, the cause of membrane changes. Because 1.5 or 2.0 M glycerol and DMSO depress nucleation temperatures to near −40°C, it would follow from this hypothesis that these additive concentrations are able to prevent such postulated changes in membrane architecture. Interesting in this regard is that these are the very concentrations of glycerol and DMSO that have been found to minimize injury when mouse embryos are frozen slowly enough to avoid internal freezing (Whittingham, et al., 1972; Leibo, 1976).

If this explanation turns out to be correct, one would have to conclude that the normal embryo contains no ice nucleators and that the architecture of the plasma membrane is such that external ice cannot grow through it. In the presence of suitable concentrations of additive, the membrane architecture remains normal during freezing, and the supercooled water in rapidly cooled cells can freeze only when the temperature falls to the homogeneous nucleation temperature. But if additive is absent or if its concentration is too low, an alteration in membrane structure occurs at relatively high subzero temperatures, and extracellular ice is able to pass through the altered region to nucleate the supercooled cytoplasm within.

SIMULTANEOUS AND STEP-BY-STEP NUCLEATION OF BLASTOMERES

Most (~80%) of the blastomeres of individual embryos were seen to flash almost simultaneously (within a second or two) during rapid cooling on the cryomicroscope stage. This result was contrary to our expectation that the nucleation of each blastomere ought to be independent of the nucleation behavior of the surrounding blastomeres. The cryomicroscopic observations suggest that ice formation in one blastomere will induce ice to form in adjacent blastomeres. One potential mechanism for the cooperative flashing behavior is the growth of ice crystals through gap junctions. Mouse embryos are known to develop gap junctions between their blastomeres at the eight-cell stage (Ducibella and Anderson, 1975). However, some (~20%) of the embryos observed during rapid cooling exhibited a "step-by-step flashing" of pairs or groups of blastomeres (see Fig. 6). Such a behavior may result from either incomplete gap junction formation or perhaps from alteration of the pores by the additive or cooling.

The Calculation of T_n

Our calculated values of T_n (Table I) assume that the protective additives had completely permeated the embryo before cooling. This really is not an assumption. The embryos were held at room temperature in the presence of the additive for sufficient time to ensure complete permeation. We determined the appropriate equilibration time by observing microscopically the time required for a complete cycle of cell shrinking and swelling after the addition of glycerol or DMSO, and then allowed an additional 5 min.

We also assumed that no dehydration of the cytoplasm occurred during cooling. To test this assumption, we used the equations of Mazur (1963 *a*) to compute the degree of shrinkage that would occur during seeding and subsequent cooling at 20°C/min. The equations require values for the water permeability and its activation energy; these were

obtained from Leibo (1980). The calculations indicate that <5% of the water in isotonic embryos would leave the cells between the seeding of the samples and the attainment of the minimum temperature.

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